OPI DATE 04/03/96 APPLN. ID 31059/95
AOJP DATE 18/04/96 PCT NUMBER PCT/AU95/00474
AU9531059

(51) International Patent Classification 6 : G01N 33/68, 33 86, C12A 1/56 — A1

(11) International Publication Number: WO 96/04560

(43) International Publication Date: 15 February 1996 (15.02.96)

(21) International Application Number: PCT/AU95/00474

(22) International Filing Date: 7 August 1995 (07.08.95)

(30) Priority Data:
PM 7313 5 August 1994 (05.08.94) AU

(71) Applicant *(for all designated States except US)*: GRADIPORE LIMITED [AU/AU]; Riverside Corporate Park, 35-105 Delhi Road, North Ryde, NSW 2113 (AU).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: EXNER, Thomas [AU/AU]; 1 Lennox Street, Gordon, NSW 2072 (AU).

(74) Agent: F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: IMPROVED ACTIVATED PROTEIN C RESISTANCE TEST

(57) Abstract

A method for determining the functional activity of activated protein C in a human plasma sample. The plasma sample is incubated with (i) exogenous reagents which activate factor V and the common pathway for blood coagulation either through factor X or by inducing the presence of thrombin in a factor V dependent manner, (ii) activated exogenous protein C; and (iii) components, such as phospholipid and calcium ions, that are necessary for efficient clotting of the plasma. The reaction is monitored to detect an end point such as the clotting time or the change in colour of a chromometric or fluorometric substrate. The end point time is compared with that obtained by a normal blood sample under the same conditions and/or with the plasma sample but without the addition of activated protein C. A decrease in clotting time as compared with normal blood is indicative of an impairment of or resistance to activated protein C.

Improved Activated Protein C Resistance Test

Field of the Invention

The present invention relates to an improved test for protein C resistance in the blood coagulation system of patients.

Background to the Invention

Known mechanisms for blood coagulation thrombosis and haemostasis are well described in International Patent Publication WO91/01382 the contents of which are incorporated herein by reference.

It is known from International Patent Publication WO93/01261 and publications by Bertina et al 1994 and Dahlback et al 1995 that the risk of thrombosis in patients with a mutant factor V molecule known as the Leiden variant, or with activated protein C impairment for some other reason, may be determined by activating the coagulation system in a plasma sample and incubating the sample with activated protein C in what has come to be known as an activated protein C impairment, impedance or resistance test. There are precedents for this test in which impairment of activated protein C has been detected in patents with acquired thrombophilia (Mitchell et al, 1986 and Amer et al, 1988).

A substrate conversion reaction rate may be determined by the clotting time or by the time required for the conversion of a chromogenic substrate to a coloured product. The conversion rate obtained is compared with values obtained in the absence of activated protein C and also with results for normal plasma samples. If the clotting time is not sufficiently prolonged by activated protein C, it indicates that the individual from which the sample is derived may be at a higher-than-normal risk of thrombosis.

Disclosure of the Invention

The present inventor has found that if factor V is specifically activated by an exogenous reagent in addition to activation of the common pathway through factor X the test for activated protein C resistance may be made more sensitive and specific than previously known tests. The present inventor has also found that a better specificity is obtained when a complex factor X activator is used together with the factor V activator. A similar result is achieved if prothrombin is activated to thrombin by a factor V dependent activator in the presence of a factor V activator.

The present invention consists in a method for determining the functional activity of protein C in a human plasma sample, comprising the steps of

(a) incubating the plasma sample with:

(i) exogenous reagents which activate factor V and which activate the common pathway of the blood coagulation mechanism through factor X or by inducing the presence of thrombin in a factor V dependent manner,

(ii) activated exogenous protein C, and

(iii) components, such as phospholipid and calcium ions, that are necessary for efficient clotting of the plasma samples;

(b) monitoring a reaction indicative of the potential rate of coagulation of the plasma sample; and

(c) comparing the potential rate of coagulation detected in step (b) with the equivalent rate determined for a normal patient, or comparing the potential rate of coagulation detected in step (b) with the equivalent rate determined for the plasma sample in the absence of activated exogenous protein C, and determining the functional activity of the free protein C from one or other of those comparisons.

In a preferred form of the invention the patient's plasma sample is pre-incubated with an exogenous activator for factor V prior to the initiation of clotting. The exogenous activators for both factors X and V are most preferably derived from snake venom. In one embodiment of the invention both the factor V and the factor X activators are derived from Russell's viper. The factor X activator is preferably derived from the venom of Russell's viper (Vipera russelli) and other immunologically cross-reactive species. A preferred factor V activator derives from Naja nivea and other immunologically cross-reactive species. The snake venoms may either be used in a diluted but unfractionated form which contributes to the simplicity of the test or, preferably, may be used in a fractionated form utilising isolated venom components.

Rather than directly activating factor X with an exogenous reagent one may also obtain an improvement over the known activated protein C test by utilising an exogenous reagent that induces in the plasma the presence of thrombin in a factor V dependent manner. In this aspect of the invention factor V dependent prothrombin activators such as those from certain Australian Notechis and Pseudonaja venoms, such as Pseudonaja textilis, Notechis scutatus and Oxyuranus scutellatus, may be used. The use of this system bypasses factor X and all factors above it thereby making the test more specific than that based on Russell's viper venom alone. The use of additional venom-derived factor V activators is desirable exactly as described above for the Russell's viper venom activated system which involves factor X activation.

Addition to the incubation mixture of a factor V activator improves the sensitivity of the test. As used in this specification a factor V activator is taken to be a compound or material which activates factor V in a manner

that makes it susceptible to degradation or deactivation by activated protein C. It is known that there are compounds which will cleave factor V in a way that renders it active but not in a form degradable by activated protein C. Such activators are not within the scope of this invention. The term activated protein C is taken to mean any compound having the functional activity of natural human activated protein C in degrading factor Va.

In the conventional APTT test and other currently used tests factor V is activated to FVa to a variable degree only by feedback from thrombin and factor Xa. These substances became present at a high level only near the clotting end point. In the method of the present invention factor V activation is a defined step induced by exogenous activator such as those derived from Russell's viper and from Naja (Cobra) venoms especially Naja nivea. Activated protein C destroys only activated factor V, not native factor V. As activated factor V is formed to a greater degree with the combination of activators present in this preferred form of the invention the test is more specific for activated protein C impedance caused by abnormal factor V.

It has recently been proposed that a major factor in activated protein C resistance is an abnormal factor V molecule which cannot be degraded by activated protein C. The importance of the present test can thus be seen in this genetic or hereditary condition. Moreover, it is clear that acquired resistance or impedance to activated protein C i.e., separate from inherited FV (Leiden), may be important in a number of clinical settings, including pregnancy, autoimmune diseases and lupus erythematosus, where antibodies may impair activated protein C function, and especially in combination with other thrombotic risk factors such as low protein S activity.

It is believed that the direct activation of factor X provides a surprising improvement over the known systems which typically are based on activated partial thromboplastin time (APTT) or prothrombin time (PT). In these known tests substantially the whole of the blood coagulation mechanism is stimulated either through the extrinsic or the intrinsic pathway. The present inventor has found that by the direct activation of factor X, particularly using snake venom derived factor X activators, and most particularly that found in Russell's viper venom and other immunologically cross-reactive species, the sensitivity of the test is improved. It is to be understood that in carrying out the invention it would be possible to merely add a factor V activator to an APTT or a PT-based activated protein C resistance test system. While this procedure is not particularly preferred it would yield the benefits of the present invention over prior art methods.

In one embodiment of the invention the components with which the patient's plasma are to be incubated are combined into a single mixture by the use of suitable surfactants, particularly non-ionic detergents. Such a single mixture preferably also contains supplemental components such as suitable buffers and preservatives. In addition the mixture preferably contains polybrene or another similar agent to reverse the effect of any heparin that may be present in the test samples. The incubation mixture preferably also contains relatively high levels of phospholipid at high ionic strength to overcome non-specific inhibitors such as lupus anticoagulants that may be present in the plasma sample.

Another complicating feature in test plasmas may be the defect caused by oral anticoagulants. Many such patients may already be on oral anticoagulant treatment which affects

the clotting tests currently used to assess activated protein C resistance.

The conventional method for minimising such interference, by mixing test plasmas with factor V deficient plasma, also works well with the Russell's viper venom (RVV) based activated protein C impedance method according to this invention. However, a more simple and less expensive method is preferred. To make the RVV-based activated protein C impedance test described here less affected by oral anticoagulant treatment, it is preferred to correct such defects by the inclusion of the vitamin K dependent clotting factors specifically depleted by such treatment. For the RVV-based test according to this invention, it is only necessary to include factors II, X and protein S. An example of a suitable source is the so-called Beriplex concentrate (Behringwerke, Germany). Addition of protein S is desirable to make the test more specific for activated protein C resistance due to factor V (Leiden) defect.

The method according to the present invention is preferably carried out as a 2-step or multistep procedure. This allows the possibility of investigating the mechanism of activated protein C resistance in a variety of clinical conditions. In this case activated protein C is preincubated for 1-5 minutes with the test plasma in the optional presence of the factor V venom activator and supplementary clotting factors II, X and protein S. Immediately thereafter a clotting reagent, sensitive to the factor V level remaining, preferably a dilute RVV-based reagent, is added and the time to a clotting endpoint is determined. If the method according to the present invention is carried out as a multi-step incubation it would be possible to add exogenous protein C and an exogenous reagent which transforms protein C into activated protein C rather than adding activated protein C itself. It has been

found that when the method is carried out using a single incubation mixture the calcium ions that must be present to induce blood clotting interfere with the activation of the exogenous protein C by the A. Contortrix-, and related species-,derived protein C activator, but not by the activation induced by the thrombin-thrombomodulin combination.

The detection system for monitoring the potential rates of change within the coagulation system may be a clotting time assay or a chromometric or fluorometric assay using an appropriate synthetic substrate. Such detection systems are well known and described in the patent specifications referred to in the introductory portions of this specification.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will be described with reference to the following examples and the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows the effect of human activated protein C on a number of clotting tests;

Figure 2 shows a comparison of traditional APTT-based activated protein C with that based on the method according to this invention on a number of patient samples;

Figure 3 shows an outline of the one and two stage methodologies for a method according to the present invention;

Figure 4 shows the relative sensitivities of one and two stage dilute DRVV tests according to this invention for activated protein C;

Figure 5 shows screening tests for factor V activators among a number of crude snake venoms;

Figure 6 shows gel filtration studies of Naja nivea venom; and

Figure 7 shows the effect of preincubating the activated protein C and factor V activator with normal and abnormal plasma prior to carrying out dilute RVV-based activated protein C resistance tests.

Best Method for Carrying out the Invention

Effect of human APC on various tests

Activated human protein C (APC) (0.5-2µg/ml) is mixed with factor V activator from Naja nivea venom (1-20 µg/ml) and then preincubated with an equal volume of test plasma for 1-5 min at 37°C. A prewarmed proprietary dilute Russell's viper venom-based reagent "LA-CONFIRM" (developed as a lupus anticoagulant resistant clotting time reagent and available from Gradipore Ltd., P.O. Box 1865, Macquarie Centre, North Ryde, NSW 2113, Australia) is then added to start the coagulation process. Comparative clotting tests are carried out in exactly the same way but without APC present and/or using pooled normal plasma.

All kit tests were carried out following the instructions of the manufacturers.

The effect of varying APC concentrations in several tests for APC resistance is shown in Figure 1. In each case the clotting times were determined on pooled normal human plasma. The index tests were carried out using COATEST-APTT reagent specifically used in the APC-resistance kit marketed by CHROMOGENEX and made according to Australian Patent Application 21980/92. Pure human APC from Haematologic Technologies Inc, USA was added to the calcium chloride used in this test. The APTT prolonged in a non-linear fashion, not exceeding 120 sec even with 4µg/ml APC present.

When APC was added to DRVVT (LA-CONFIRM) a much more linear response was obtained (Fig. 1) and the clotting time exceeded 150sec with less than 2µg/ml APC. When the DRVVT

reagent was modified to contain only the factor X activator isolated from Russell's viper venom (RVVFXa CT)(obtained from American Diagnostica Inc, USA and not containing the factor V activator) a reduced sensitivity to APC was obtained. This clearly shows that factor V activation by the crude Russell's viper venom improves the sensitivity to APC and is a desirable component of the test system.

The identical test based on Oxyuranus Scutellatus (Taipan) venom (OXYURA/CT) at a concentration of 5×10^{-6}% w/v added to the LA-CONFIRM formula devoid of Russell's viper venom showed prolongation by APC though less than that induced in either the APTT or DRVV-based tests.

Comparison of Various APC Resistance Tests

Six members of a family believed to have familial thrombophilia were tested with the dilute Russell's viper venom-based APC resistance test in comparison with the Coatest APTT-based method. Results expressed as APC resistance ratios are shown in Fig. 2 in comparison with various normal healthy blood donors. No individuals appeared to be abnormal using the Coatest method and a cut-off of 2.0. The DRVVT-based method, however, revealed that 4 of the 6 family members were clearly abnormal. Also 3 of the 19 normal individuals appeared abnormal with this test. The abnormal results are circled in Fig. 2. DNA analysis of all of the apparently abnormal individuals subsequently confirmed the presence of an arginine 506 to glutamine mutation, conferring APC resistance i.e. the Leiden factor V deficiency. The lower APC resistance ratios in affected members determined with the DRVVT-based test relative to mean normal clearly shows it to be a more sensitive test than the APTT-based Coatest method.

Methodology

The initially-described one-stage method in which APC was premixed with the DRVVT reagent is shown schematically in Fig. 3. The mixed reagent showed some instability especially at room temperature and a 2-stage method was devised (Fig. 3). In this the APC is added separately from the DRVVT reagent. The APC reagent has been found in this case to be more stable. The APC is preferably mixed with a factor V activator derived from Naja venoms in the same schematic diagram. The method resembles a traditional APTT format and is thus easily adaptable for automation.

Comparison of 1- and 2-stage DRVVT tests for APC resistance:

Fortuitously it was found that the 2-stage method was more sensitive to APC concentration, expressed in Fig. 4 as the level of APC in the final clotting mixture volume. Numerous other studies have shown that the 2-stage method provided similar discrimination of FV (Leiden) subjects from normal whilst being technically more reliable. In the procedure illustrated in Fig. 3 factor V activator can be added with the APC or at any earlier stage to enhance this discrimination (with less APC being required for similar long clotting times among normals). Thus the two stage method is most sensitive to activated protein C and this is even more enhanced when an additional venom FV activator is included.

Screening for FV activators:

Crude venoms often contain procoagulants complicating the search for anticoagulant components. Figure 5 shows the effect of various Elapid venoms reported to contain factor V activators on an APC resistance test (Gerads *et al.* 1992). Most of these shortened the clotting time due to exogenous activators of coagulation. Significant activated protein C enhancing activity, presumably due to factor V activator, was detectable in unfractionated Naja nivea venom. Naja

nivea solutions prolonged LA-CONFIRM results on normal plasma in the presence of APC suggesting that a factor V activator, stimulating factor Va inactivation by APC, may have been present. This venom was fractionated by gel filtration. It is expected that fractionation of the other venoms would reveal components which could act as FV activators for use in the present invention.

Gel Filtration:

Gel filtration of Naja nivea venom on Sephadex G, 50 and testing of factors confirmed the presence of a powerful APC-enhancing component. As shown in Fig. 6 the peak anticoagulant activity occurred at fraction number 37. Analysis by SDS - polyacrylamide gel electrophoresis revealed a main protein band at 60,000 ± 10,000 molecular weight.

Mechanism of the APC-enhancing activity:

Fig. 7 shows the effect of varying the preincubation time for a venom factor V activator (fraction 37) derived from Naja nivea with pooled normal plasma (PNP) and abnormal plasma (ABN) derived from a patient with the FV Leiden genetic trait both with activated protein C (+ APC) and without activated protein C (-APC). The preincubated plasma were then tested for clotting time using a DRVVT-based APC in a time dependent manner and resistance test. These results confirm that the factor V activator from Naja nivea venom enhances the sensitivity of normal plasma to prolongation by APC in a time dependent manner and to a much greater degree than with factor V (Leiden) plasma.

References

BERTINA RM, KEULEMANS BPC, KOSTER T, et al
Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 369; 64-67, 1994.

DAHLBACK B - Inherited Thrombophilia:
Resistance to activated protein C as a pathogenic factor of venous thromboembolism. Blood 85; 607-614, 1995

MITCHELL CA, ROWELL JA, HAU L, et al
Fatal thrombotic disorder associated with an acquired inhibitor of protein C. N. England J. Med. 317; 1638-16, 1987

AMER L, KISIEL W, SEARLES RP, WILLIAMS JRC
Impairment of the protein C anticoagulant pathway in a patient with systemic lupus erythematosus, anticardiolipin antibodies and thrombosis. Thromb. Res. 57, 247-1990

GERADS I, TANS G, YUKELSON LY, et al. Activation of bovine factor V by an activator purified from the venom of Naja oxiana. Toxicon 30; 1065-1079, 1992.

CLAIMS:

1. A method for determining the functional activity of protein C in a human plasma sample, comprising the steps of

(a) incubating the plasma sample with:

(i) exogenous reagents which activate factor V and activate the common pathway of the blood coagulation mechanism through factor X or by inducing the presence of thrombin in a factor V dependent manner,

(ii) activated exogenous protein C, and

(iii) components, such as phospholipid and calcium ions, that are necessary for efficient clotting of the plasma samples;

(b) monitoring a reaction indicative of the potential rate of coagulation of the plasma sample; and

(c) comparing the potential rate of coagulation detected in step (b) with the equivalent rate determined for a normal patient, or comparing the potential rate of coagulation detected in step (b) with the equivalent rate determined for the plasma samples in the absence of activated exogenous protein C, and determining the functional activity of the free protein C from one or other of those comparisons.

2. A method as claimed in claim 1 in which the plasma sample is pre-incubated with an exogenous activator for factor V prior to the initiation of blood clotting.

3. A method as claimed in claim 1 in which exogenous reagent which activates the common pathway of the blood coagulation mechanism is a reagent that directly activates factor X.

4. A method as claimed in claim 3 in which the factor X activator is an activator derived from a snake venom.

5. A method as claimed in claim 4 in which the factor X activator is derived from the venom of Russell's viper

(Vipera russelli) and other immunologically cross-reactive species.

6. A method as claimed in claim 1 in which the factor V activator is derived from a snake venom.

7. A method as claimed in claim 6 in which the factor V activator is derived from Naja nivea or another immunologically cross-reactive species.

8. A method as claimed in claim 7 in which the factor V activator is a 60,000 ± 10,000 MW protein fractionated from the venom of Naja nivea.

9. A method as claimed in claim 1 in which the common pathway of the blood coagulation mechanism is activated by inducing the presence of thrombin in a factor V dependent manner using a snake venom.

10. A method as claimed in claim 9 in which the snake venom is derived from a species of the genus Notechis, Oxyuranus or Pseudonaja.

11. A method as claimed in claim 1 in which the functional activity of active protein C is determined by comparing the potential rate of coagulation detected in step (b) with the equivalent rate determined for a normal patient.

12. A method as claimed in claim 1 in which the monitored reaction indicative of the potential rate of coagulation of the plasma is the actual plasma clotting time.

13. A method as claimed in claim 1 in which the monitored reaction indicative of the potential rate of coagulation of the plasma is the conversion of a chromometric or fluorometric substrate by an enzyme generated through the clotting process.

14. A method as claimed in claim 1 in which the plasma sample is also incubated with phospholipid at high ionic strength to neutralise or overcome lupus anticoagulants.

15. A method as claimed in claim 1 in which the plasma sample or the activated protein C is incubated with exogenous vitamin K dependent clotting factor to supplement those depleted in a patient by oral anti-coagulant treatment.

16. A method as claimed in claim 15 in which the clotting factors are selected from the group comprising factor II, factor X, protein S and mixtures thereof.

17. A method as claimed in claim 2 in which the sample plasma is pre-incubated with the exogenous activator for factor V for 1 to 5 minutes and/or with activated protein C before clotting is initiated.

FIGURE 1

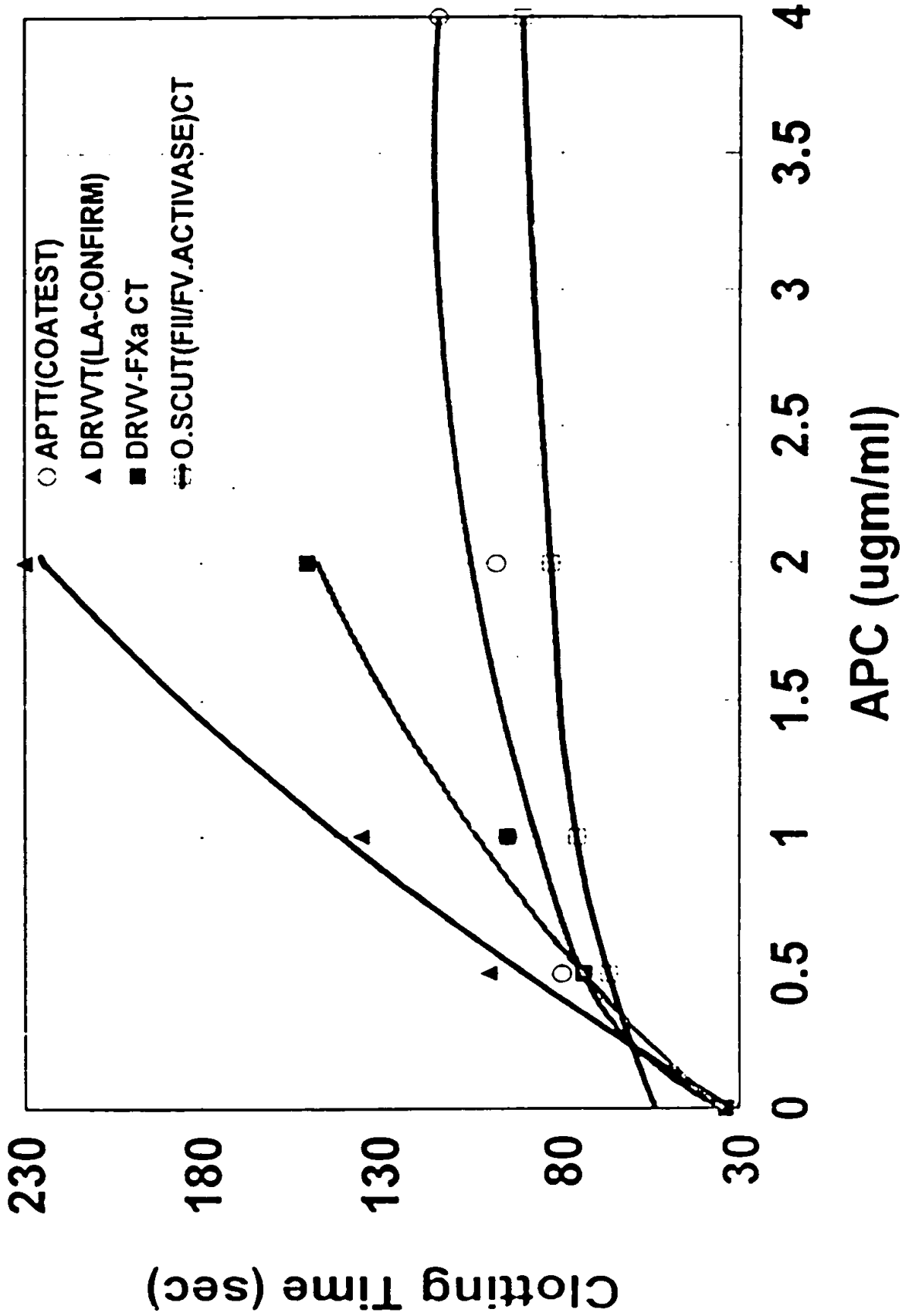

Clotting Time (sec)
230
180
130
80
30
0
0.5
1
1.5
2
2.5
3
3.5
4
APC (ugm/ml)
APTT(COATEST)
DRVVT(LA-CONFIRM)
DRVV-FXa CT
O.SCUT(FII/FV.ACTIVASE)CT

FIGURE 2

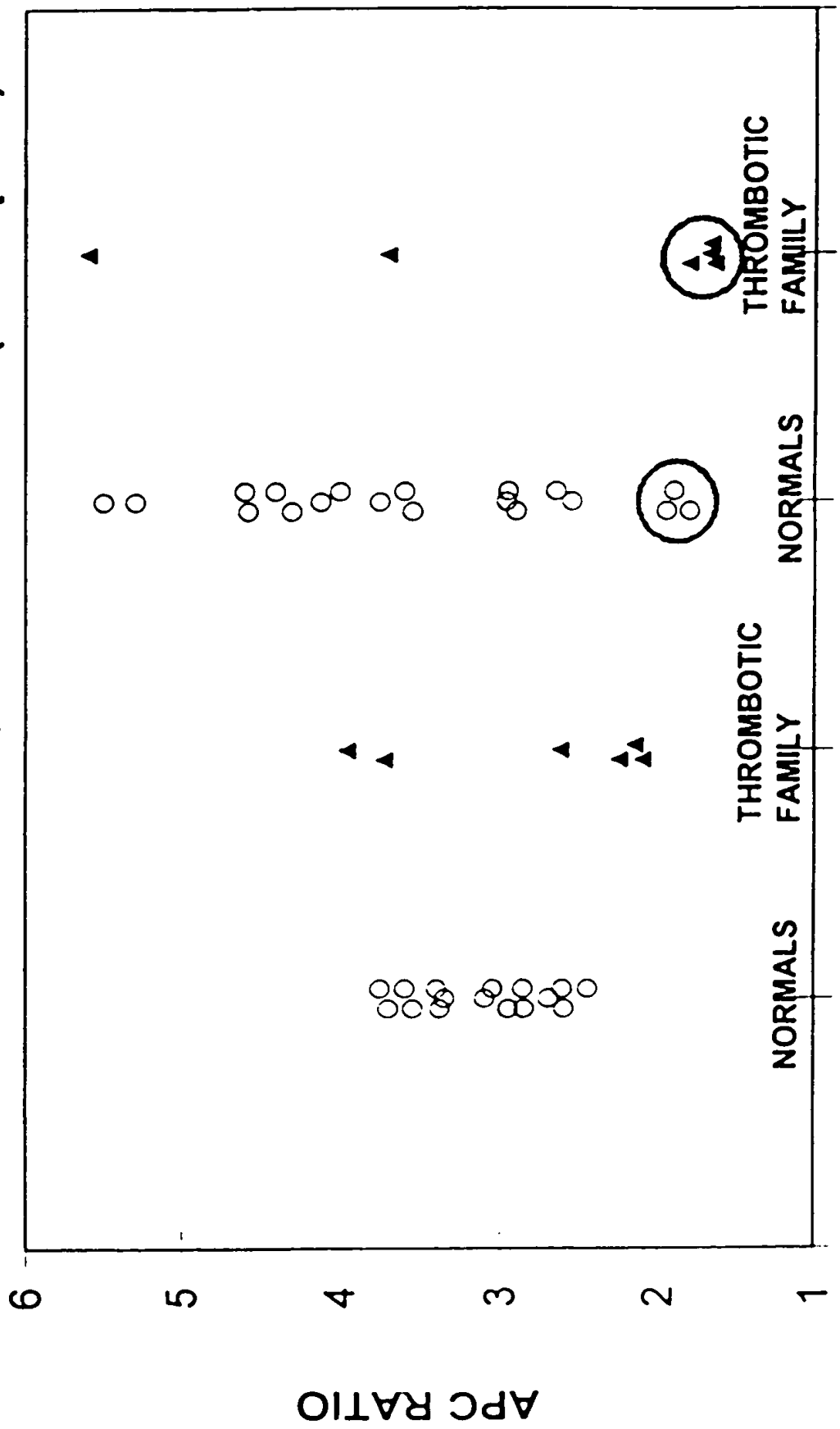

APTT (Coatest)
DRVVT (Gradipore)
APC RATIO
6
5
4
3
2
1
NORMALS
THROMBOTIC FAMILY
NORMALS
THROMBOTIC FAMILY

FIGURE 3

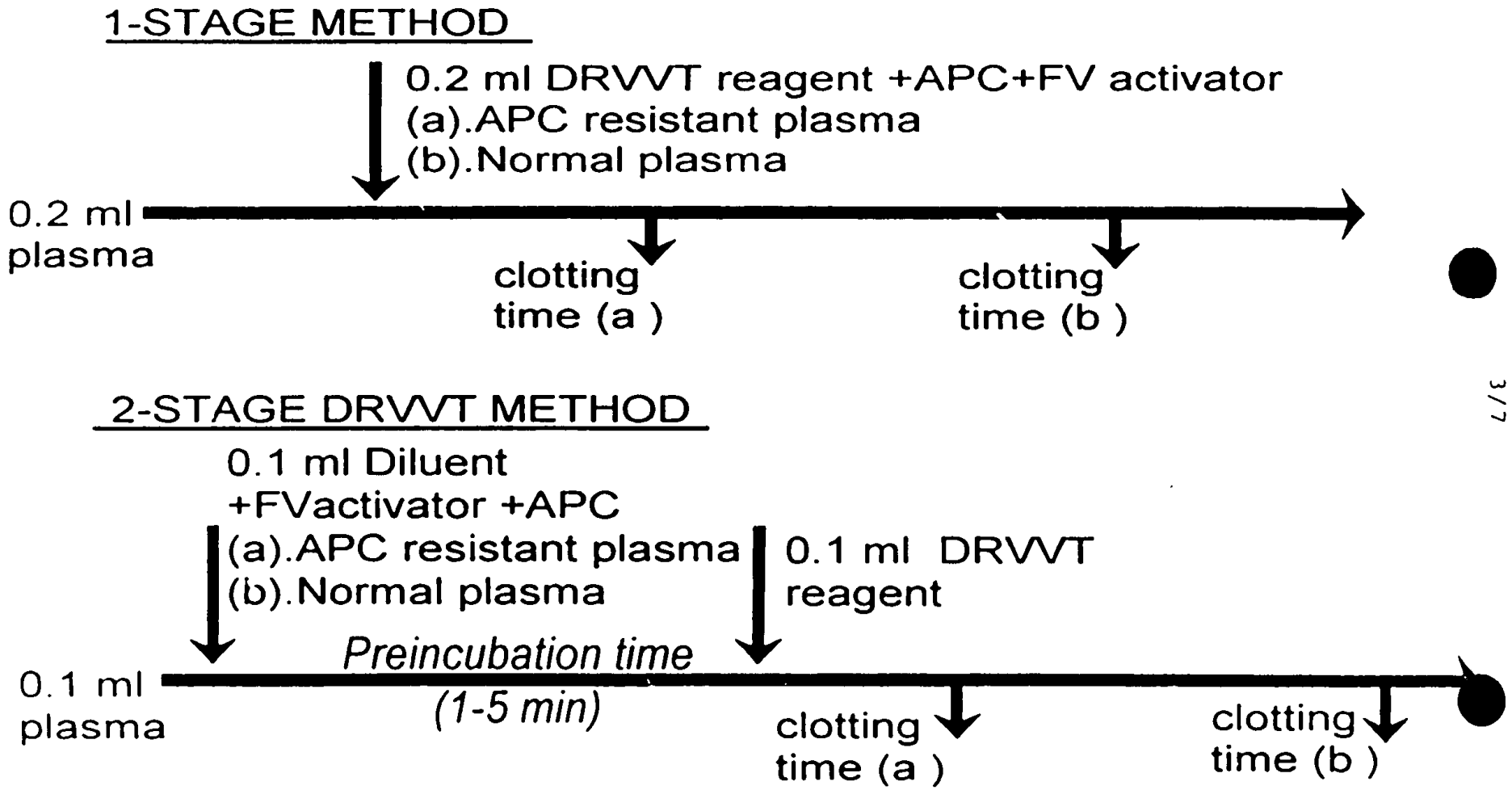

1-STAGE METHOD
0.2 ml DRVVT reagent +APC+FV activator
(a).APC resistant plasma
(b).Normal plasma
0.2 ml plasma
clotting time (a)
clotting time (b)
2-STAGE DRVVT METHOD
0.1 ml Diluent +FVactivator +APC
(a).APC resistant plasma
(b).Normal plasma
0.1 ml DRVVT reagent
Preincubation time
(1-5 min)
0.1 ml plasma
clotting time (a)
clotting time (b)

FIGURE 4

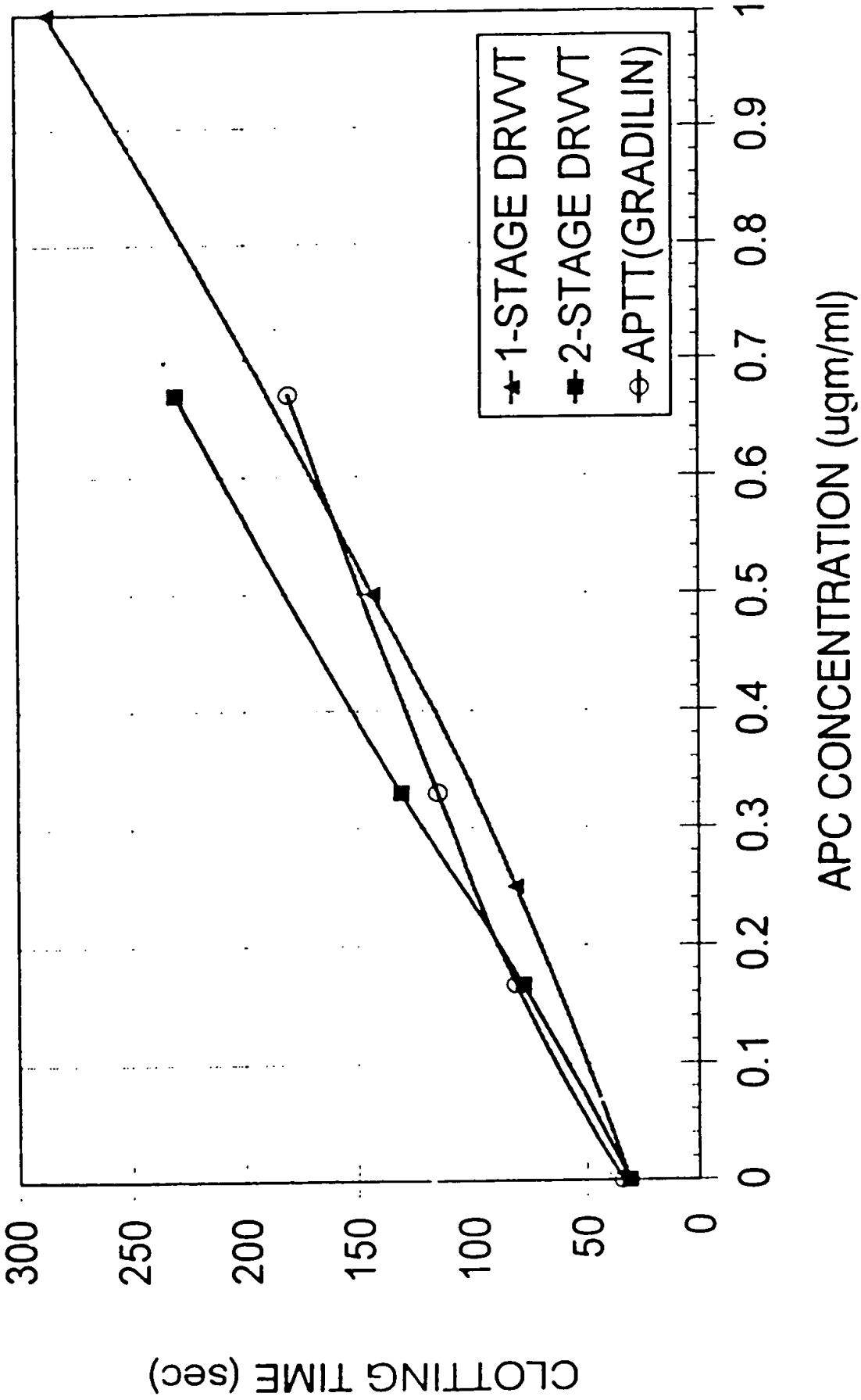

1-STAGE DRVVT
2-STAGE DRVVT
APTT(GRADILIN)
CLOTTING TIME (sec)
APC CONCENTRATION (ugm/ml)
0
50
100
150
200
250
300
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1

FIGURE 5

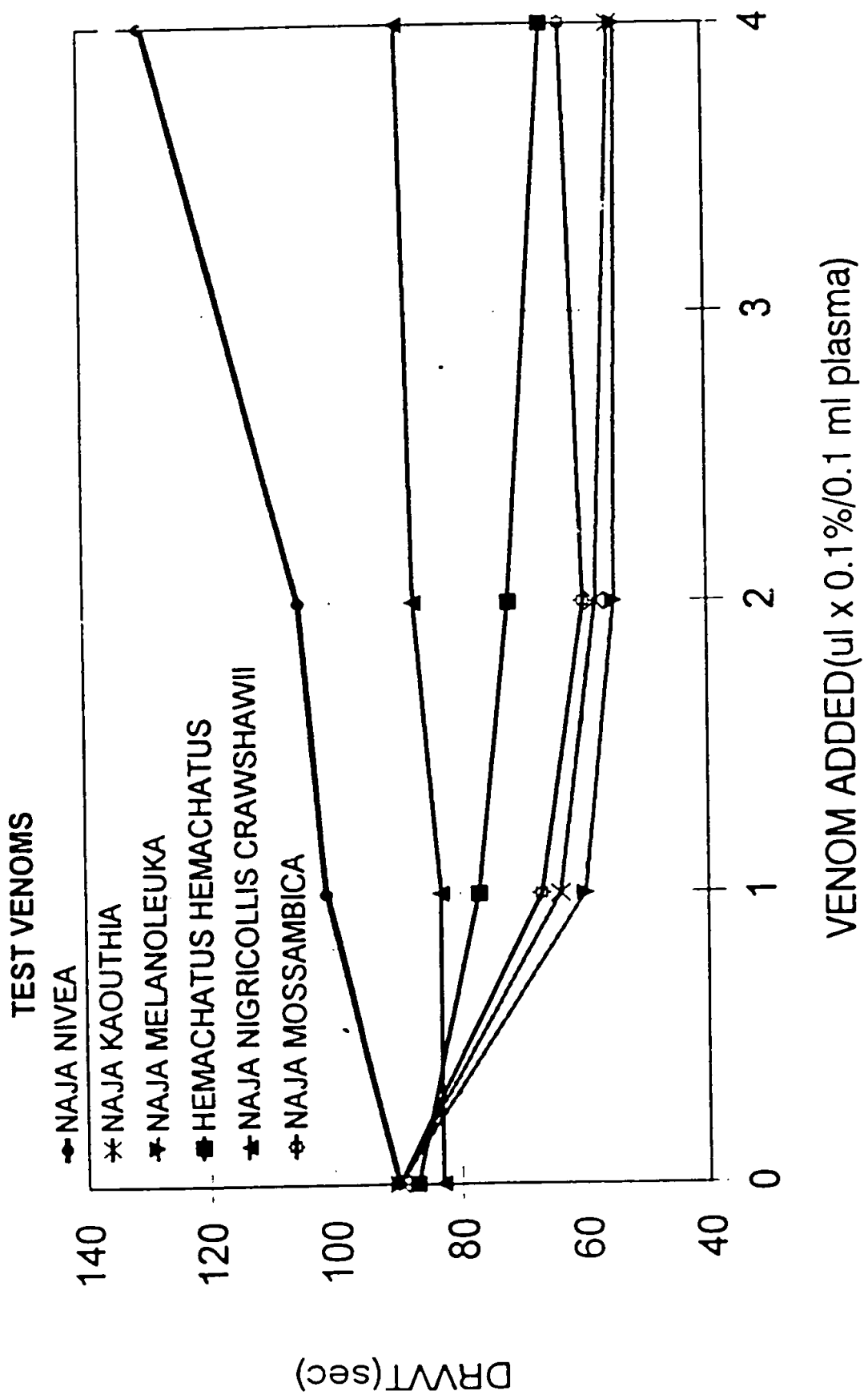

TEST VENOMS
NAJA NIVEA
NAJA KAOUTHIA
NAJA MELANOLEUKA
HEMACHATUS HEMACHATUS
NAJA NIGRICOLLIS CRAWSHAWII
NAJA MOSSAMBICA
DRVVT(sec)
140
120
100
80
60
40
0
1
2
3
4
VENOM ADDED(ul x 0.1%/0.1 ml plasma)

FIGURE 6

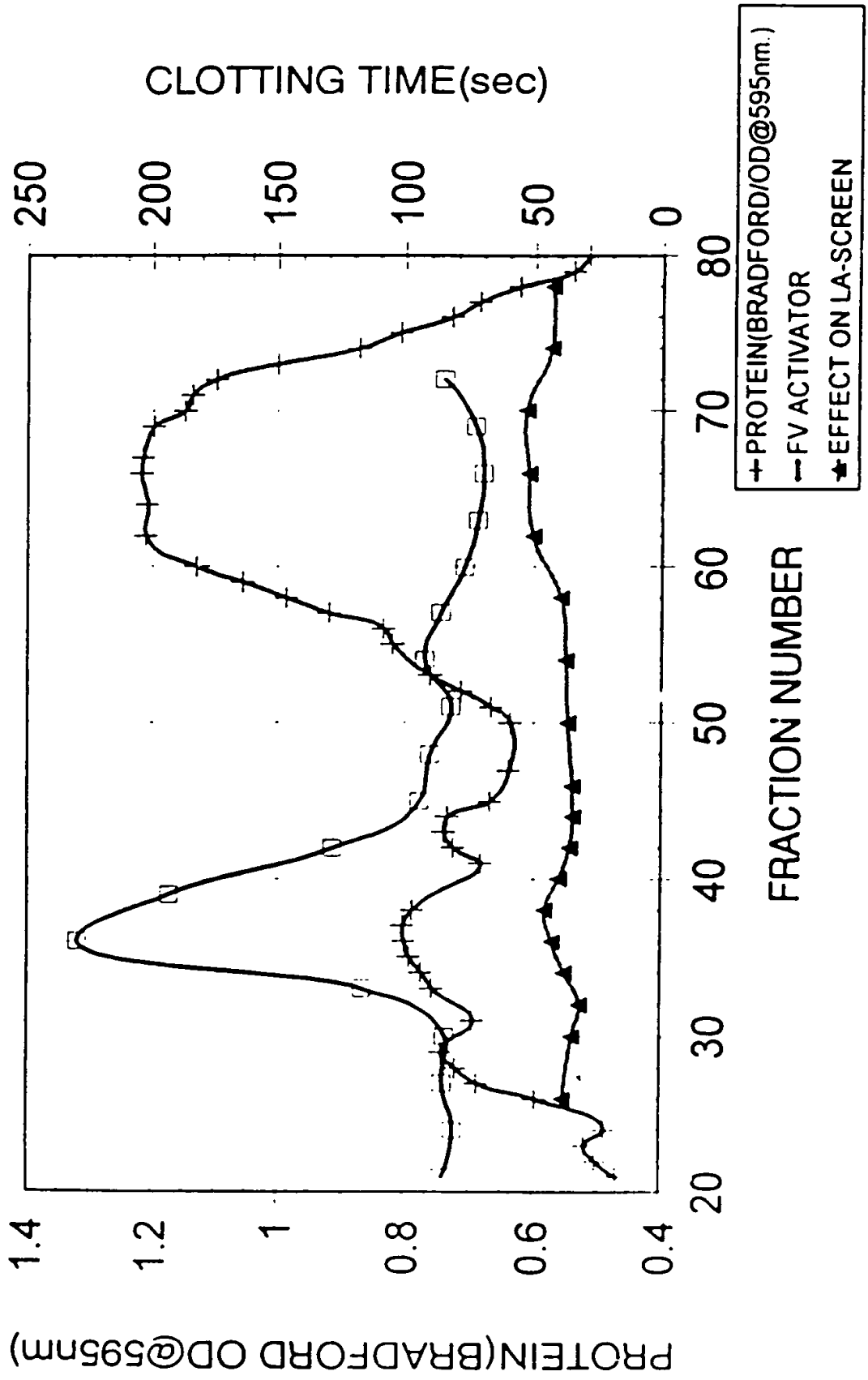
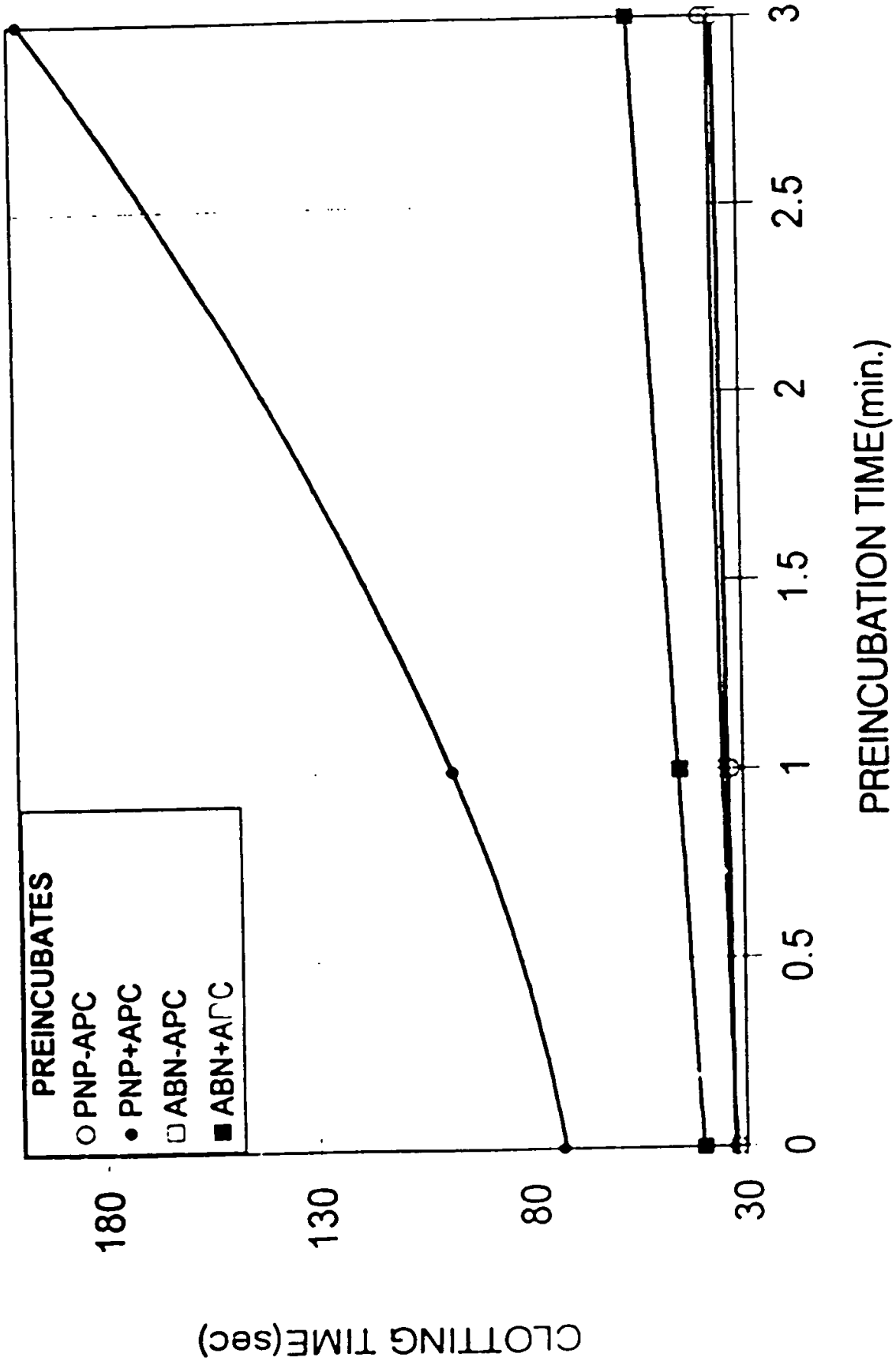

CLOTTING TIME(sec)
250
200
150
100
50
0
PROTEIN(BRADFORD OD@595nm)
1.4
1.2
1
0.8
0.6
0.4
FRACTION NUMBER
20
30
40
50
60
70
80
PROTEIN(BRADFORD/OD@595nm.)
FV ACTIVATOR
EFFECT ON LA-SCREEN

FIGURE 7


PREINCUBATES
○ PNP-APC
● PNP+APC
□ ABN-APC
■ ABN+APC
180
130
80
30
CLOTTING TIME(sec)
0
0.5
1
1.5
2
2.5
3
PREINCUBATION TIME(min.)

INTERNATIONAL SEARC. REPORT

International Application No
PCT/AU 95/00474

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6] G01N 33/68, 33/86, C12A 1/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
DERWENT WPAT database; Chemical Abstracts Service, file CASM;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPM

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, USPM databases: Keywords used were protein () C, fact () V or proaccelerin # or pro()accelerin # or labile()factor CA database: Keywords used as above plus 9/cc, 7/cc, 1/cc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO, 93/10261 (DAHLBACK, Bjorn) 27 May 1993 (see whole document, expecially page 4).	1, 2, 3, 11-14, 16, 17
X	AU,A, 60533/90 (Kabivitrum AB) 7 February 1991 (see whole document)	1, 3, 11, 13, 14

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
7 November 1995

Date of mailing of the international search report
14 NOVEMBER 1995

Name and mailing address of the ISA/AU
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION
PO BOX 200
WODEN ACT 2606
AUSTRALIA Facsimile No (06) 285 3929

Authorized officer
Carmela Monger
Telephone No (06) 283 2486

Form PCT/ISA/210 (second sheet) (July 1992) cooky

TERNATIONAL SEARCH REPORT
nformation on patent family members

International Application No
PCT/AU 95/00474

This Annex lists the known "A" publication level patent family members relating to the patent documents cited n the above-mentioned international search report. The Australian Patent Office is in no way liable for these articulars which are merely given for the purpose of information

Patent Document Cited in Search Report		Patent Family Member			
WO	9310261	AU	21980/92	EP	608235
AU	60533/90	EP	486515	WO	9101382

END OF ANNEX